

A comparison of gene transfer methods in human dendritic cells

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Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) for the initiation of antigen-specific T-cell activation. DCs may be highly enriched from peripheral blood-adherent leukocytes by short-term (7-day) culture in the presence of interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor. Various methods of gene transfer were studied, including DNA/liposome complexes, electroporation, CaPO_4 precipitation, and recombinant adenovirus (AdV) vectors. Low levels of expression were obtained with the physical methods tested. In contrast, AdV vectors expressing luciferase, β -galactosidase, IL-2, and IL-7 all readily transduced human DCs. Increasing levels of gene expression were observed over a range of multiplicity of infection (MOI) of 10:1 to 10,000:1, with transduction efficiencies exceeding 95% at higher MOI. Although levels of maximal gene expression in DCs were significantly lower than those obtained using human tumor cell lines, IL-2 and IL-7 production of up to 5×10^2 ng/ 10^6 DC were achieved. These results suggest that AdV vectors are a promising vehicle for genetically engineering human DCs.

Key words: Interleukin-2; dendritic cells; adenovirus; liposomes; transgene.

Dendritic cells (DCs) are potent antigen-presenting cells¹⁻⁸ with the capacity to acquire and process antigen,^{9,10} migrate to lymphoid organs, and stimulate antigen-specific T cells to proliferate.^{3,11} DCs express high levels of major histocompatibility complex (MHC) class I and II, as well as important costimulatory molecules.^{4,12-15}

Until recently, efforts to study and modify their function were limited by their rare presence in peripheral blood, constituting only 0.05% to 0.3% of peripheral blood mononuclear cells (PBMCs).^{11,16} However, we⁴ and others¹⁷⁻²⁰ have described methods by which large numbers of potent DCs can be propagated from peripheral blood or bone marrow with the aid of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 or GM-CSF and tumor necrosis factor. As observed with fresh DCs, cytokine-propagated DCs express high levels of MHC, B7-1, B7-2, CD40, and adhesion molecules.^{4,21-25} More importantly, these cells retain the capacity to stimulate antigen-specific immunity.^{4,26-29} Indeed, using a murine model, Mayordomo et al.¹⁸ recently observed that bone marrow-derived DCs pulsed with tumor-associated peptides could elicit an antigen-specific T-cell response against tumors and lead

to subsequent protection from a lethal tumor challenge. The ready availability of cultured DCs and their capacity to stimulate potent and antigen-specific antitumor immunity make them an attractive target for gene therapy. Transferring tumor antigen genes into DCs should empower them with the ability to intrinsically process these antigens and efficiently stimulate antitumor responses. Likewise, cytokines such as IL-2, IL-7, and IL-12 are known to enhance the growth and effector function of stimulated T cells. Expression of these cytokines by gene-modified DCs should significantly enhance the T-cell response to tumor antigen presentation.

In this study, we demonstrate the ability to genetically engineer DCs *in vitro* to express several different foreign proteins, including IL-2 and IL-7, after gene transfer by a variety of methods. Recombinant E1-deleted adenovirus (AdV) vectors were far superior to any of the physical methods of gene transfer used (lipofection, electroporation, or CaPO_4 precipitation). As in tumor cell lines, gene expression in DCs was a linear function of multiplicity of infection (MOI), and functionally important levels of cytokine production were achieved.

MATERIALS AND METHODS

Preparation of dendritic cells

Human DCs were prepared by culturing the adherent fraction of PBMCs for 7 days in GM-CSF and IL-4, as previously described.^{4,20} In brief, PBMCs from healthy donors were washed, resuspended in complete medium (RPMI 1640 (GIBCO-BRL, Grand Island, NY) with 10% human AB serum (Gemini Bioproducts) and penicillin-streptomycin-fungizone,

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(GIBCO-BRL)] and allowed to differentially adhere to 25-cm² tissue culture flasks (Costar) by culturing 3 to 4 × 10⁶ cells in 8 mL, for 2 hours at 37°C. The nonadherent cells were then removed by gentle rinsing, and the remaining adherent cells were cultured in 8 mL complete medium supplemented with 800 U/mL human recombinant GM-CSF (Sigma, St. Louis, Mo) and 500 U/mL human recombinant IL-4 (R & D Systems, Minneapolis, MN) for 6 to 8 days. DCs were recovered at the end of the incubation by vigorous washing of the flasks to remove all of the cells that were not firmly adherent. This cell population routinely contained 60% to 80% DCs and was used directly for gene transfer studies.

Recombinant AdV vectors

A seed viral stock of a recombinant AdV vector (AdVLacZ), which contains the *Escherichia coli* β -galactosidase (lacZ) reporter gene with a nuclear localization sequence, was generously provided by Drs Robert Moen and Bruce Trapnell (Genetic Therapy Inc, Gaithersburg, Md). A seed viral stock of another recombinant AdV vector (AdVLuc), which contains the firefly, *Photinus pyralis*, luciferase (Luc) reporter gene was generously provided by Dr Michael Barry (University of Texas, Southwestern, Dallas, Tex). AdVRR5, AdVLacZ, and AdV-Luc are E1-deleted, replication-deficient adenovirus type-5 (Ad-5) vectors. AdVRR5 has no reporter gene. The Luc and lacZ reporter genes were inserted into the former E1 site of the respective vectors. The lacZ gene was inserted in reverse orientation with respect to the Ad-5 sequences. Both reporter genes are transcribed from the cytomegalovirus (CMV) enhancer/promoter and terminate with an SV40 polyadenylation sequence.

We constructed an AdV vector (AdVIL-2) that contains the human IL-2 complementary DNA (cDNA; generously provided by Dr Joseph Rosenblatt, UCLA Medical Center) using modifications of methods kindly provided by Dr Trapnell. Briefly, the AdVIL-2 was prepared through an *in vitro* recombination event in 293 embryonic renal cells between a shuttle plasmid, pAC-CMVpLpA (generously provided by Dr Robert Gerard, University of Texas, Southwestern), which contains the human IL-2 cDNA insert within the first 3.2 kb of the E1-deleted Ad-5 genome, and the pJM17 plasmid (generously provided by Dr Barry), which contains the entire E1-deleted Ad-5 genome. Likewise, we have constructed a recombinant AdV vector (AdVIL-7), which contains the human IL-7 cDNA (kindly provided by Dr. Graeme Dougherty, Terry Fox Laboratories, Vancouver, British Columbia). The IL-2 and IL-7 genes were inserted within the polylinker at the former E1 site and are driven by the CMV promoter/enhancer. Clones of AdVRR5, AdVIL-2, and AdVIL-7 were obtained by limiting dilution analysis of the ability of the media to induce a cytopathic effect on 293 fresh cells and confirmed by IL-2 or IL-7 enzyme-linked immunosorbent assay (ELISA), respectively. Viral stocks were then obtained by amplification of the 293 cells followed by CsCl purification, dialysis, and storage as a glycerol (10% vol/vol) stock at -80°C. The titer of each viral stock was routinely between 10⁹ and 10¹³ plaque-forming units (pfu)/mL by plaque assay on 293 cells. Contamination with wild-type recombinant AdV was assessed for each viral stock by plaque assay on HeLa cells and was consistently negative.

Plasmids

The pCMVLuc plasmid, which has the firefly, *Photinus pyralis*, Luc reporter gene, was constructed by recombining the pXP1 plasmid, generously provided by Dr Steve Nordeen (University of Colorado, Denver), and pUC18 (Pharmacia, Alameda,

Calif), which has a CMV promoter/enhancer. pCMVIL-2 was prepared in a similar manner using a shuttle plasmid, pAC-CMVpLpA, and human IL-2 cDNA.

Gene transfer techniques

Cultured human DCs were directly compared with melanoma cell lines (M202 and M207) that were established in our laboratory. Both DCs and melanoma cell lines were subjected to the following gene transfer techniques: cationic lipofection, electroporation, calcium phosphate precipitation, or transduction with either control AdV vector, or AdV vectors containing IL-2, IL-7, luciferase, or β -galactosidase.

Transfection of cells with cationic liposomes

Several different commercially available cationic liposome preparations including LipofectAMINE, Lipofectin, Lipofect ACE (GIBCO-BRL), and DOTAP (Boehringer-Mannheim) were used according to the manufacturer's published protocols. Transfection of both melanoma and DCs was performed in suspension in media composed of serum-free OptiMEM (GIBCO-BRL) for 4 hours at 37°C in 5% CO₂, after which time RPMI-1640 supplemented with 30% serum (human AB serum for DC and fetal bovine serum for melanoma) and antibiotics was added to each transfection condition to bring the total serum concentration to 10%. After 24 hours, cells were given additional culture medium. Cells were transfected using several different amounts of liposomes and plasmid DNA in an attempt to discover the optimal concentrations of each. Liposome amounts ranged from 1 to 50 μ g, and DNA amounts ranged from 2 to 50 μ g.

Electroporation of cells

Both DCs and melanoma were electroporated in RPMI-1640 supplemented with 20% fetal bovine serum at a concentration of 2 × 10⁷ cells/mL using a BioRad Gene Pulser electroporator at voltages ranging from 100 to 400 V. DNA was added to cells, which were then incubated on ice for 10 minutes before electroporation. After electroporation, cells were incubated on ice for another 10 minutes before their return to culture. Cells were returned to culture in 5 mL RPMI-1640 supplemented with 10% serum (AB serum for DCs and fetal bovine serum for melanoma) until assay at 48 hours.

Calcium phosphate precipitation

For each transfection condition, 10 μ g of DNA was added to 25 μ L 1.25 mol/L CaCl₂ and 215 μ L water to a final volume of 250 μ L. This was then added to 250 μ L 2× HBS to bring total volume to 500 μ L. Air was bubbled into the mixture for 30 seconds. The mixture was incubated for 30 minutes at room temperature. The mixture was added to cells and allowed to incubate at 37°C with 5% CO₂ for 4 hours. After this time, the DNA mixture was aspirated from cells, and cells were fed with fresh culture medium.

Transduction of cells with AdV vectors

Transductions were performed in infection media composed of either RPMI-1640 supplemented with 2% human AB serum and antibiotics (DCs) or RPMI-1640 medium supplemented with 2% fetal bovine serum and antibiotics (M202 and M207). Cells were transduced in suspension with AdV at various MOIs (MOI = 1–10,000) at 37°C in 5% CO₂ for 2 hours, after which time the cells were fed by the addition of 3 vol of culture medium and returned to culture. Cell counts were determined

manually using a hemacytometer and viability assessed by trypan blue exclusion. No additional cytokines were added to the medium after transduction.

Mixed lymphocyte reaction

DCs or adherent PBMCs from the same donor were plated in various dilutions (corresponding to APC:T-cell ratios of 1:10 to 1:80) in triplicate in a 96-well round-bottom microtiter plate in 100 μ L complete medium per well. The plate was irradiated at 3000 rad from a cesium source, and then 10^5 nonadherent responder PBMC from a different donor were added to each well in a volume of 100 μ L. The cells were cultured at 37°C for 6 days and then pulsed overnight with 0.5 μ Ci/well 3 H-thymidine (Amersham, Arlington Heights, Ill). The cells were harvested onto glass fiber disks in a PhD harvester (Cambridge Technology, Cambridge, Mass), and the 3 H-thymidine uptake was determined by scintillation counting. Data presented are an average of the triplicate wells in one of three similar experiments performed with three different nonadherent cell donors and two different DC and adherent PBMC donors.

Luciferase assay

Luciferase was assayed using the Monolight 1500 luminometer from Analytical Luminescence Laboratory. Cells were pelleted, lysed using 200 μ L luciferase extraction buffer (1% Triton X-100, 25 mmol/L glycyl-glycine, 15 mmol/L $MgSO_4$, and 4 mmol/L EGTA) and vortexed. A quantity of 250 μ L luciferase assay buffer (25 mmol/L glycyl-glycine, 15 mmol/L $MgSO_4$, 4 mmol/L EGTA and 15 mmol/L KPO_4) was pre-loaded into 12 \times 75-mm analytical luminescence cuvettes. A quantity of 100 μ L of each cell lysate was added to each cuvette and placed into the luminometer. Luciferin substrate was added by the luminometer.

IL-2 and IL-7 ELISA

IL-2 was assayed using a standard kit (Genzyme Diagnostics, Cambridge, Mass) according to the protocol published by the manufacturer. IL-7 was assayed using a standard kit (R & D Systems) according to the protocol published by the manufacturer. Samples were run in duplicate with corresponding cytokine standards and controls. Experimental values were determined by linear regression analysis.

β -galactosidase staining with X-gal

Cell supernatants were aspirated from each well. Cells were fixed by adding 2 to 3 mL X-gal fixative solution [phosphate buffered saline (PBS) pH 7.4, 2% (vol/vol) formaldehyde, 0.2% (vol/vol) glutaraldehyde] to each well and incubated at 4°C for 5 minutes. Wells were washed twice with 1 \times PBS (pH 7.4). X-gal staining solution (PBS pH 7.4, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 2 mmol/L $MgCl_2$, and 1 mg/mL X-gal (added just before staining)) was added to wells and incubated at 37°C with 5% CO_2 for 6 to 24 hours. At the end of the incubation period, wells were washed multiple times with PBS to remove all X-gal crystals; and left in 3 mL PBS until photographs were taken.

Transduction efficiency was assessed using the LacZ reporter gene. Cells were transduced using an MOI of AdVLacZ ranging from 1 to 10,000. After 48 hours, cells were fixed as above, and the percentage of blue cells was assessed using a light microscope and calculating the number of blue cells with DC morphology as compared with the total number of DCs per high power field. Culture-derived DCs were easily identified by

their large size, granular cytoplasm, and ruffled, stellate membranes.

Flow cytometry

As a more sensitive method of quantifying the proportion of cells transduced by an AdV infection, lacZ expression was assessed by flow cytometry using the lacZ Fluoreporter kit (Molecular Probes Inc, Eugene, Or). Briefly, cells were transduced with AdVLacZ at an MOI of 1000 in 12 \times 75 mm polystyrene tubes to ensure maximal numbers of DCs in the assay. Twenty-four hours after transduction with AdVLacZ, cells were pelleted, washed in PBS with 2% AB serum, and stained with pre-conjugated primary antibodies CD14 (PE), B7-2 [phycoerythrin (PE)] or human leukocyte antigen HLA-DR (Per CP) for 30 minutes on ice. Cells were then washed twice in PBS, pelleted, and resuspended at 10^7 cells/mL in staining medium [PBS supplemented with 4% (vol/vol) fetal bovine serum, 10 mmol/L HEPES, pH 7.2] and placed on ice. A 100- μ L cell aliquot was prewarmed at 37°C for 10 minutes and then loaded with warm (37°C) 2 mmol/L fluorescein di- β -D-galactopyranoside (FDG) in 8:1:1 H_2O /DMSO/EtOH (vol/vol). Loading with FDG was stopped after 1 minute by adding ice-cold staining medium. Cells were washed twice after FDG loading with ice-cold PBS, pelleted, and resuspended in 200 μ L of PBS plus 0.2% paraformaldehyde. Cells were kept on ice until flow cytometry, which was performed within 30 minutes of cell preparation on a FACScan apparatus (Becton Dickinson, Mountain View, Calif). DCs were identified by their large size and complexity as previously described.⁴ Endogenous β -galactosidase activity was inhibited, when necessary, by the addition of 300 mmol/L chloroquine to the cell suspensions before FDG loading.

To determine the effects of transduction on the expression of DC cell surface markers, sham-transduced or AdVLacZ transduced DC were cultured for 24 hours, stained with FDG as described, and counterstained with monoclonal antibodies to CD3, CD13, CD14, CD19, CD11c, CD40, intracellular adhesion molecule (ICAM)1, MHC class I, MHC class II, B7-1 (CD80), and B7-2 (CD86) and analyzed by flow cytometry. Antibodies to CD13, CD40 (Caltag Laboratories, San Francisco, Calif) CD3, and CD19 (Becton Dickinson) were pre-conjugated to FITC. Antibodies to B7-1 (Becton-Dickinson), CD14 (Caltag Laboratories), B7-2, MHC class I, CD11c, and

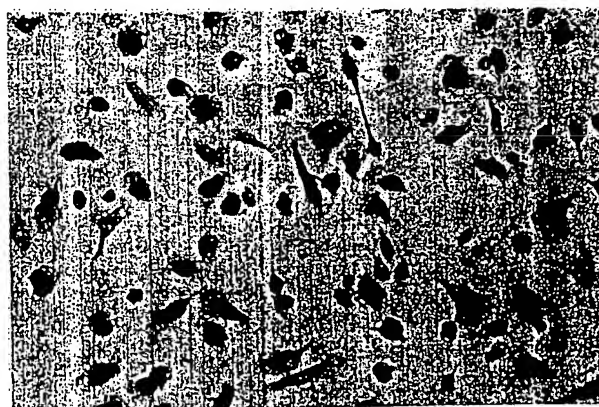


Figure 1. Photomicrograph of DCs at 100 \times magnification. DCs that have been transduced with AdVLacZ at an MOI of 1000 and then stained days later for the presence of β -galactosidase.

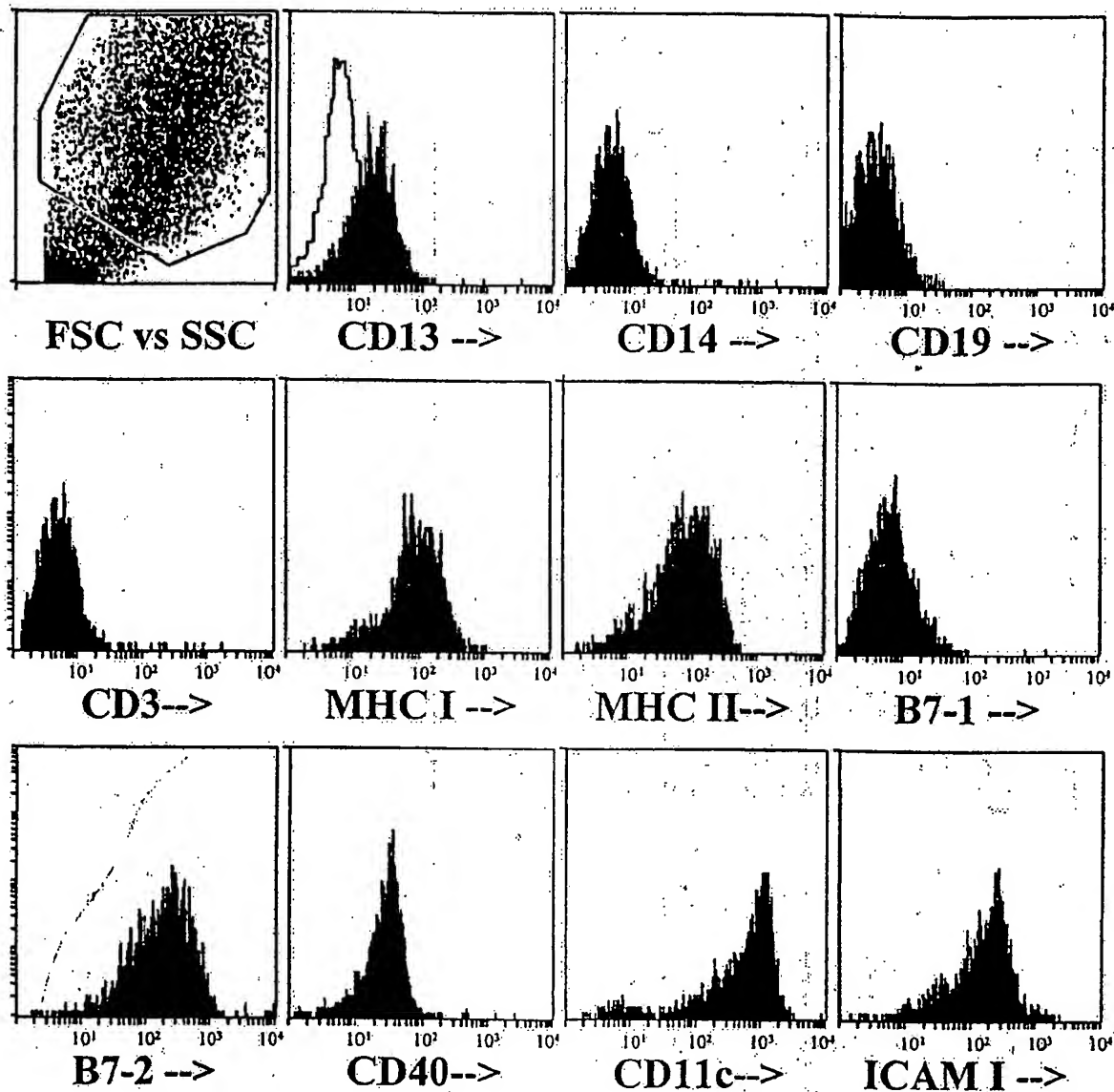


Figure 2. Adherent PBMCs cultured in GM-CSF and IL-4 for 1 week give rise to a combination of lymphocytes and DCs (upper left panel). Gating on cells with a large (high forward scatter), granular (high side scatter) profile constituting 83% of the recovered cells demonstrates a population with the phenotype of cultured DCs. These cells stained for the myeloid marker CD13 (unstained control depicted in white) but were negative for expression of CD14 (macrophages), CD19 (B cells), and CD3 (T cells). Cultured DCs express low levels of B7-1 and high levels of HLA-ABC, HLA-DR, B7-2, CD40, CD11c, and ICAM-1. Results are from a sample representative experiment.

ICAM1 (PharMingen, San Diego, Calif) were pre-conjugated to PE. Antibodies to MHC class II were pre-conjugated to PerCP (Becton Dickinson). Ten thousand cells were evaluated for each marker and results expressed as the mean linear fluorescence of the positively staining cells.

RESULTS

Preparation of DCs were obtained from adherent PBMCs that were differentiated for 6 to 8 days in tissue

culture in the presence of IL-4 and GM-CSF as previously described.⁴ The identity of these cells as DCs was supported by (1) morphology (using light microscopy, DCs appeared as large cells with ruffled, stellate membranes)^{14,20} (Fig 1); (2) phenotype (by flow cytometry, these large cells lacked expression of CD3, CD14, and CD19 and did express high levels of CD13, MHC class I, MHC class II, B7-1, B7-2, CD11c, CD40, and ICAM1) (Fig 2); and (3) function (these cells generated strong allogeneic mixed lymphocyte reaction (MLR) response

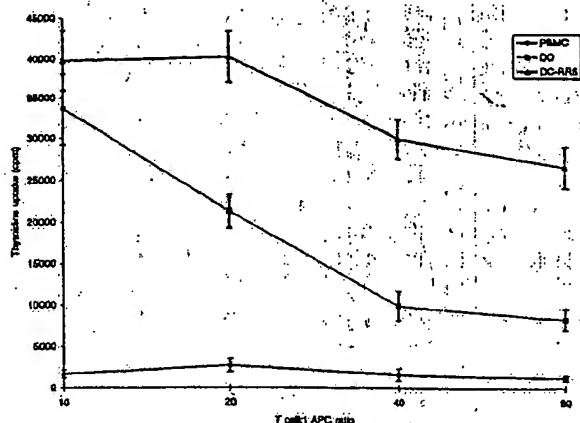


Figure 3. Cultured DCs and Adv-transduced DCs are superior to PBMCs as T-cell stimulators in the MLR. From 1×10^4 to 1.25×10^5 DCs or fresh adherent PBMCs were plated per well, in triplicate. DC-RR5 were transduced with the Adv at an MOI of 1000. Responder allogeneic nonadherent PBMCs were then plated at 1×10^5 cells/well. T-cell stimulation was assayed by thymidine uptake after 8 days.

in vitro compared with fresh adherent PBMCs from the same donor (Fig 3). The resultant cell population at 7 days varies from 50% to 80% DCs (Fig 2).

Physical transfection methods

DC populations were transfected with a Luc expression plasmid using liposomes, electroporation, and CaPO_4

precipitation. A range of transfection conditions and liposome preparations were used. Melanoma cell lines, which are readily transfected by all methods tested, were used as positive transfection controls. Generally low levels of Luc expression were noted with electroporation and lipofection in DCs when compared with a human melanoma cell line (Fig 4). CaPO_4 precipitation was not effective for gene transfer into DCs and led to excessive cell death. Consideration was given to the possible phenotypic and functional changes of 7-day cultured DCs and their ability to acquire and process antigen compared with fresh DCs.^{11,18,25,30-34} Several attempts were made to lipofect fresh DCs (harvested after the initial 2-hour adherence) using various concentrations of DNA and liposomes. None of these was any more successful than the lipofection using 7-day cultured DCs. In contrast, transduction with a recombinant adenovirus vector bearing the luciferase reporter gene (AdvLuc) at MOIs of 10:1 and 100:1 generated levels of expression orders of magnitude higher.

Adenovirus-mediated gene transfer

DCs were transduced using AdvIL-2, AdvIL-7, AdvLuc, or AdvLacZ vectors (Fig 5). Cells were infected with MOI ranging from 1:1 to 10,000:1. A melanoma cell line was used as a positive control. DCs consistently produced two to four orders of magnitude less gene product than did melanoma cells at similar MOI, regardless of which Adv vector was used. Transduction efficiency of AdvVs was assessed using AdvLacZ. At 100:1 MOI, less than 30% of DCs were transduced. However, greater than 95% of DC were transduced at

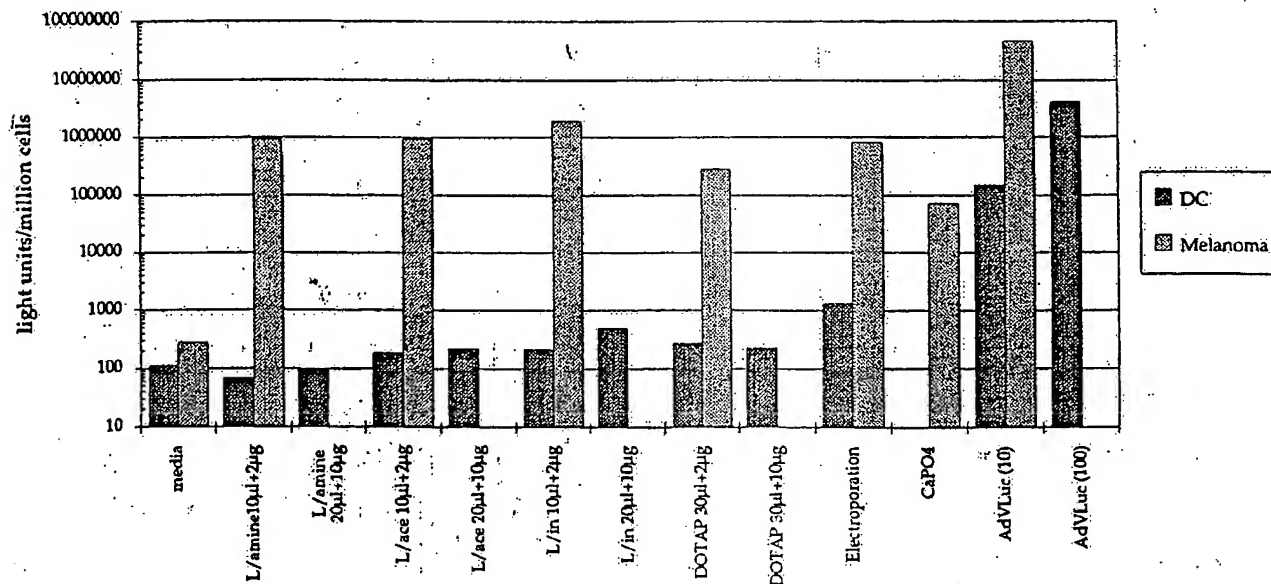


Figure 4. Comparison of gene transfer methods in DCs versus melanoma. Cells were transfected/transduced with the luciferase reporter gene by one of four methods: (1) lipofection using one of several commercially available liposome preparations (LipofectAMINE, LipofectACE, Lipofectin, or DOTAP), (2) electroporation, (3) calcium phosphate precipitation, or (4) AdvLuc infection. Melanoma cell lines were not transfected with the higher concentrations of liposome preparations. AdvLuc at an MOI of 100 was not performed with melanoma cells.

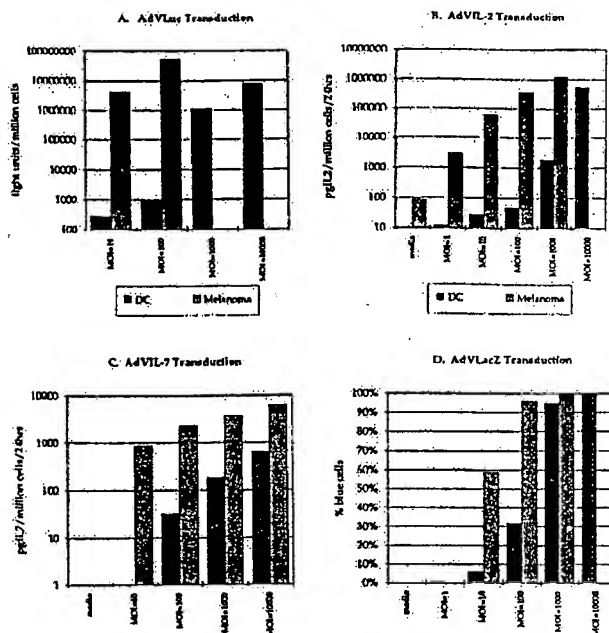


Figure 5. AdV transduction of DCs and a melanoma cell line using several genes. Four different AdV vectors expressing luciferase (A), IL-2 (B), IL-7 (C), or β -galactosidase (D) were used to transduce DCs or a melanoma cell line. Luciferase, IL-2, or IL-7 activity was assayed after 24 hours as described (A, B, C). Cells were stained for β -galactosidase as described (D). Melanoma cell lines were not transduced using higher MOIs due to the cytopathic effect.

1000:1 MOI, and 100% were transduced at 10,000:1 MOI. The cytopathic effect of a virus load of 1000:1 and 10,000:1 that is observed in melanoma cells (40% cell death and 100% cell death, respectively) was not observed in DCs, with sustained viability of over 90% after 7 days in culture. When transduced at 100 times higher MOI, DCs produced similar levels of gene product as melanoma. DC transduced with AdVIL-2 produced high levels of IL-2 over the course of 7 days, approximately 500 ng/10⁶ cells/24 hours (Fig 6).

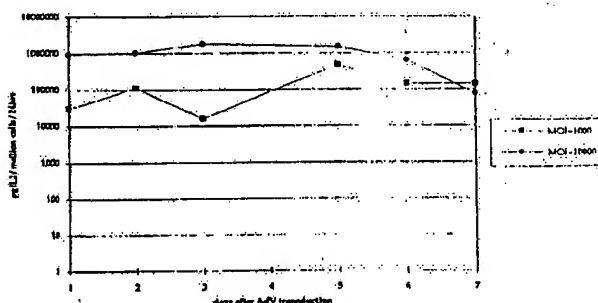


Figure 6. Time course of IL-2 expression in DCs after transduction with AdVIL-2 at an MOIs of 1000 and 10,000. The medium was changed daily so that levels of IL-2 production reflect each 24-hour period.

To confirm that the transduced cells were in fact DCs, we used flow cytometry to stain transduced DCs for β -gal expression via FDG loading while counterstaining for relevant markers (Fig 7). The effect of AdV transduction on phenotype and function of DCs was studied. Using flow cytometry, DCs transduced with AdVLacZ at 1000:1 MOI were compared with nontransduced DCs with regard to expression of cell surface markers. Table 1 demonstrates that high-MOI AdV infection does not alter the ability of DCs to express relevant cell surface markers. Virally transduced DCs retained their increased ability to stimulate T-cell proliferation in the MLR (Fig 3). In some cases, transduced DCs were even more effective APCs than were their untransduced counterparts, possibly due to simultaneous presentation of adenoviral antigens.

DISCUSSION

DCs are the most potent APCs known. Using recently developed methods, it is now possible to culture large numbers of DCs from the adherent fraction of PBMCs.^{4,17-20} These culture-derived DCs can be pulsed with exogenous antigens and are far superior to PBMCs in their ability to stimulate antigen-specific T cells.^{4,5,7,27,30-38} When primed with tumor antigens, they have been shown to induce tumor regression and anti-tumor immunity in murine models of established cancer.¹⁸ We hypothesize that genetically engineering DCs to express a given tumor antigen would offer several potential advantages over peptide pulsing. Intrinsic processing of encoded proteins would result in multiple peptide fragments capable of associating with the cell's own MHC molecules. In this way, gene-modified DCs would likely stimulate multiple T-cell clones, each one reactive to a different epitope from the same tumor antigen. In addition, intrinsic processing automatically selects for those peptides capable of binding to the cell's own MHC, thereby obviating the need to carefully identify and synthesize different peptides to be used with every possible MHC subtype.

Building on these precedents, we investigated several different approaches for engineering DCs to express foreign genes, including lipofection, electroporation, calcium phosphate precipitation, and transduction with replication-deficient AdVs. We used β -galactosidase-, luciferase-, and cytokine-reported genes because of the unique techniques by which each of these genes can be assayed for their level of expression. Of the approaches studied, AdV transduction was clearly the most efficient method for expressing transgenes in human culture-derived DCs. Transgene expression was up to 1000-fold more efficient using AdV vectors than the expression produced by any other method tested. Viral transduction did not significantly alter the expression of important DC cell surface markers, such as MHC, B7-1, B7-2, CD40, or a variety of adhesion molecules. More importantly, virally transduced DCs retained their potent

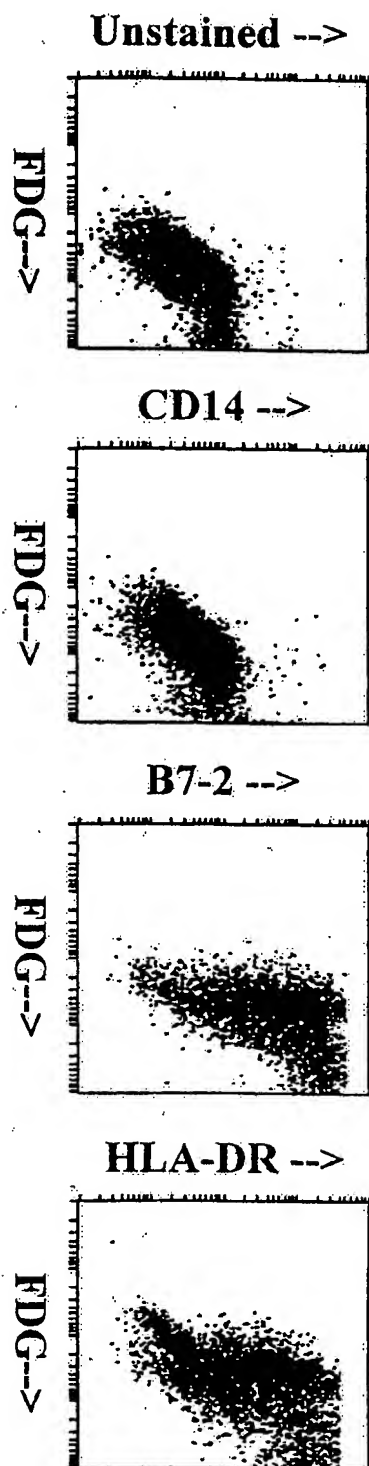


Figure 7. FACS analysis demonstrates efficient transduction of DCs. Adherent PBMCs cultured in GM-CSF and IL-4 were transduced with AdVLacZ at an MOI of 1000. The cells were stained for β -gal expression by FDG loading and simultaneously for the expression of either CD14, B7-2, or HLA-DR. The DC population was gated as described in Fig 2 and evaluated for expression of these markers.

Table 1. A Comparison of Cell Surface Markers on DC With and Without AdVLac2 Transduction at MOI of 1000 Using Flow Cytometry

Cell Surface Marker	Mean Linear Fluorescence (MLF)	
	Nontransduced DC	Transduced DC MOI = 1000
CD14	0	0
CD19	0	0
HLA-ABC	1501.23	2065.04
HLA-DR	1604.09	1375.33
B7-1	46.8	48.05
B7-2	3083.11	3013.17
CD11c	7539.3	4344.68
CD13	334.17	286.37
CD40	558	718.41
ICAM-1	2969.19	2066.87

T-cell stimulatory activity as measured by the MLR assay.

Of the physical methods tested, electroporation produced the highest levels of transgene expression. However, as stated, expression was still minimal compared with the expression achieved when melanoma tumor cells were used as the targets. In our hands, lipofection induced levels of gene expression that were barely detectable above background. However, it appears that even this low level of expression may be functionally effective in DCs. In a recent report, DCs generated by the same GM-CSF/IL-4 method were transfected with the tyrosinase gene using lipofectin reagent.³⁹ Although only low levels of transgene expression were observed, as in our study, tyrosinase-transfected DCs were able to cluster with and activate T cells. We did not investigate whether transgene expression produced by any of our techniques was sufficient to allow DCs to stimulate antigen-specific T cells. Further studies of this are clearly warranted.

At high MOI, recombinant AdV vectors produced high levels of transgene expression in our melanoma tumor cells. However, when comparing the same MOI, gene expression achieved in DCs was always two to four orders of magnitude less than that produced by melanoma cells. The results were the same with all four reporter genes. Human DCs appear to be relatively refractory to infection by AdV, with MOIs of 1000:1 or greater being required to achieve transduction efficiencies of greater than 90%. However, human DCs are similarly resistant to the cytopathic effects of this virus, allowing high MOI to be used without untoward cell death. DCs transduced by AdVLuc at an MOI of 10,000 generated luciferase activity at a level of 10^7 light units/million cells. DCs transduced by AdVIL-2 at an MOI of 10,000 produced IL-2 in the range of 500 to 1000 ng/million cells/24 hours. AdVIL-2-transduced DCs continued to produce high levels of IL-2 for a week in tissue culture, and the level of IL-2 transgene expression in DCs was appreciable for up to 2 weeks in some experiments (data not shown), but precipitously dropped off thereafter due to *in vitro* cell death. Simi-

larly, the production of IL-7 by AdVIL-7-transduced DCs approached 1 ng/million cells/24 hours at an MOI of 10,000.

The local production of cytokines by DCs could provide an important adjunct for T-cell activation in cancer patients who are often immunosuppressed. Genetic engineering of DCs with AdV vectors would allow cells to be simultaneously transduced with more than one vector. Such an approach could provide antigen transgenes for directing immune specificity and cytokine transgenes for reversing tumor-induced immunosuppression.^{40,41} Genetically engineered DCs may provide a novel strategy for stimulating specific antitumor immunity.

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REFERENCES

1. Van Voorhis WC, Hair LS, Steinman RM, Kaplan G. Human dendritic cells: enrichment and characterization from peripheral blood. *J Exp Med*. 1982;155:1172-1187.
2. Van Voorhis WC, Valinsky J, Hoffman E, Luban J, Hair LS, Steinman RM. Relative efficacy of human mononuclear and dendritic cells as accessory cells for T cell replication. *J Exp Med*. 1983;158:174-191.
3. Steinman RM. The dendritic cells system and its role in immunogenicity. *Ann Rev Immunol*. 1991;9:271-296.
4. Kiertscher SM, Roth MD. Human CD14⁺ leukocytes acquire the phenotype and function of antigen-presenting dendritic cells when cultured in GM-CSF and IL-4. *J Leukoc Biol*. 1996;59:208-218.
5. Young JW, Steinman RM. Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4⁺ helper T cells. *J Exp Med*. 1990;171:1315-1332.
6. Inaba K, Metlay JP, Crowley MT, Steinman RM. Dendritic cells pulsed with protein antigens *in vitro* can prime antigen-specific, MHC-restricted T cells *in situ*. *J Exp Med*. 1990;172:631-640.
7. Bhardwaj N, Friedman SM, Cole BC, Nisanian AJ. Dendritic cells are potent antigen-presenting cells for microbial superantigens. *J Exp Med*. 1992;175:267-273.
8. Thomas R, Davis LS, Lipsky PE. Comparative accessory cell function of human peripheral blood dendritic cells and monocytes. *J Immunol*. 1993;151:6840-6852.
9. Nijman HW, Kleijmeer MJ, Ossevoort MA, et al. Antigen capture and major histocompatibility class II compartments of freshly isolated and cultured human blood dendritic cells. *J Exp Med*. 1995;182:163-74.
10. Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med*. 1995;182:389-400.
11. Cumberbatch M, Gould SJ, Peters SW, Kimber I. MHC class II expression by Langerhans' cells and lymph node dendritic cells: possible evidence for maturation of Langerhans' cells following contact sensitization. *Immunology*. 1991;74:414-419.
12. Freudenthal PS, Steinman RM. The distinct surface of human blood dendritic cells, as observed after an improvised isolation method. *Proc Natl Acad Sci USA*. 1990;87:7698-7702.
13. O'Doherty U, Steinman RM, Peng M, et al. Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J Exp Med*. 1993;178:1067-1078.
14. Caux C, Vanbervliet B, Massacrier C, Azuma M, Okumura K, Lanier LL, Banchereau J. B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J Exp Med*. 1994;180:1841-1847.
15. Zhou L-J, Tedder TF. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J Immunol*. 1995;154:3821-3835.
16. Thomas R, Lipsky PE. Human peripheral blood dendritic cell subsets: isolation and characterization of precursor and mature antigen-presenting cells. *J Immunol*. 1994;153:4016-4028.
17. Szabolcs P, Moore MAS, Young JW. Expansion of immunostimulatory dendritic cells among the myeloid progeny of CD34⁺ bone marrow precursors cultured with c-kit ligand, granulocyte-macrophage colony-stimulating factor, and TNF- α . *J Immunol*. 1995;154:5851-5861.
18. Mayordomo JJ, Zorina T, Storkus WJ, et al. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nature Med*. 1995;1(12):1297-1302.
19. Bernhard H, Disis ML, Heimfeld S, Hand S, Gralow JR, Cheever MA. Generation of immunostimulatory dendritic cells from human CD34⁺ hematopoietic progenitor cells of the bone marrow and peripheral blood. *Can Res*. 1995;55:1099-1104.
20. Romani N, Gruner S, Brang D, et al. Proliferating dendritic cell progenitors in human blood. *J Exp Med*. 1994;180:83-93.
21. Caux C, Massacrier C, Vanbervliet B, et al. Activation of human dendritic cells through CD40 cross-linking. *J Exp Med*. 1994;180:1263-1272.
22. Vreinec D, Zorbas M, Scollay R, et al. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med*. 1992;176:47-58.
23. Sotzik F, Rosenberg Y, Boyd AW, et al. Assessment of CD4 Expression by Early T precursor cells and by dendritic cells in the human thymus. *J Immunol*. 1993;152:3370-3377.
24. MacPherson GG. Properties of lymph-borne (veiled) dendritic cells in culture. I. Modulation of phenotype, survival, and function: partial dependence of GM-CSF. *Immunology*. 1989;68:102-107.

25. Reid CDL, Stackpole A, Meager A, Tikerpa J. Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth *in vitro* from early bipotent CD34+ progenitors in human bone marrow. *J Immunol*. 1992;149:2681-2688.
26. Langhoff E, Steinman RM. Clonal expansion of human T lymphocytes initiated by dendritic cells. *J Exp Med*. 1989;169:315-320.
27. Pancholi P, Steinman RM, Bhardwaj N. Dendritic cells efficiently immunoselect mycobacterial-reactive T cells in human blood, including clonable antigen-reactive precursors. *Immunology*. 1992;76:217-224.
28. Macatonia SE, Taylor PM, Knight SC, Askonas BA. Primary stimulation by dendritic cells induces antiviral proliferation and cytotoxic T cell responses *in vitro*. *J Exp Med*. 1989;169:1255-1264.
29. Rouse RJD, Nair SK, Lydy SL, Bowen JC, Rouse BT. Induction *in vitro* of primary cytotoxic T-lymphocyte responses with DNA encoding herpes simplex virus proteins. *J Virol*. 1994;68:5685-5689.
30. O'Doherty U, Peng M, Gezelter S, Swiggard WJ, Betjes M, Bhardwaj N, Steinman RM. Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology*. 1994;82:487-493.
31. Crowley M, Inaba K, Steinman RM. Dendritic cells are principal cells in mouse spleen bearing immunogenic fragments of foreign proteins. *J Exp Med*. 1990;172:383-386.
32. Aiba S, Katz SI. The ability of cultured langerhans cells to process and present protein antigens is MHC-dependent. *J Immunol*. 1991;146:2479-2487.
33. Bhardwaj N, Young JW, Nisanian AJ, Baggers J, Steinman RM. Small amounts of superantigen, when presented on dendritic cells, are sufficient to initiate T cell responses. *J Exp Med*. 1993;178:633-642.
34. Manca F, Pira GL, Fenoglio D, Fang SP, Habeshaw A, Knight SC, Dalglish AG. Dendritic cells are potent antigen-presenting cells for *in vitro* induction of primary human CD4+ T-cell lines specific for HIV gp120. *J AIDS*. 1994;7:15-23.
35. Cohen PJ, Katz SI. Cultured human langerhans cells process and present intact protein antigens. *J Invest Dermatol*. 1992;99:331-336.
36. Weissman D, Li Y, Ananworanich J, et al. Three populations of cells with dendritic morphology exist in peripheral blood, only one of which is infectable with human immunodeficiency virus type 1. *Proc Natl Acad Sci USA*. 1995;92:826-830.
37. Tjoa B, Erickson S, Barren R III, et al. *In vitro* propagated dendritic cells from prostate cancer patients as a component of prostate cancer immunotherapy. *Prostate*. 1995;27:63-69.
38. Mjaaland S, Fossum S. Dendritic leucocytes pulsed with monoclonal antibody-hapten conjugates elicit vigorous primary humoral responses *in vivo*. *Scand J Immunol*. 1995;41:305-308.
39. Alijagic S, Moller P, Artuc M, Jurgovsky K, Czarnetski BM, Schadendorf D. Dendritic cells generated from peripheral blood transfected with human tyrosinase induces specific T cell activation. *Eur J Immunol*. 1995;25:3100-3107.
40. Toloza EM, Hunt K, Miller AR, et al. Transduction of murine and human tumors using recombinant adenovirus vectors. *Ann Surg Oncol*. 1996 (in press).
41. Toloza EM, Hunt K, Swisher S, et al. *In vivo* cancer gene therapy with a recombinant interleukin-2 adenovirus vector. *Cancer Gene Ther*. 1996 (in press).

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